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DEVELOPMENT OF *ONCHOCERCA VOLVULUS* (FILARIOIDEA: ONCHOCERCIDAE) IN THE WEST AFRICAN BLACK FLY *SIMULIUM YAHENSE* (DIPTERA: SIMULIIDAE) IN LIBERIA

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ABSTRACT: *Simulium yahense* black flies infected with microfilaria of *Onchocerca volvulus* were kept in a defined insectary environment in Liberia, West Africa. A daily sample of infected flies was dissected for larvae developing in the thoracic muscles and examined for growth in stadial development. Microfilariae ingested by black flies transformed to the L_1 larval stage without molting. Successive larval development included molting to the L_2 stage and, finally, to the L_3 stage, which was infective in humans. The cephalic cap, consisting of a laterally located hook and central stoma, occurs in the first larval stage. The caudal appendix and the laterally located anal opening are apparent in the L_1 larva. In the L_2 stage, the cephalic cap is lost and the large circular stoma becomes surrounded with elevated flaps. The caudal appendix was lost after larvae molted to the L_3 stage, and in its place, 3 terminal papillae developed. Sense organs, such as 2 opposing phasmids and 8 papillae that were arranged into 2 circles, developed in the cephalic region of the L_3 larva. The evidence of pathological consequences due to the presence of the L_3 larva in the fly host are illustrated and discussed.

Microscopic studies of the development and morphology of *Onchocerca volvulus* in *Simulium damnosum* s.l. black flies were first described more than 70 yr ago in Sierra Leone by Blacklock (1926a, 1926b) and later in Cameroon by Duke (1968). Scanning electron microscopy (SEM) studies, which reveal morphologic detail characters in a vector, were reported for *O. volvulus* larvae by Franz and Renz (1980), who examined third-stage larvae (L_3) for identification purposes, and by Franz and Schulz-Key (1981), who showed the anterior region of 3 larval stages.

Gibson et al. (1976), Martinez-Palomo and Martinez-Báez (1977), and Matsuo et al. (1980) used transmission electron microscopy (TEM) to study the ultrastructure of *O. volvulus* microfilariae. Strote and Bonow (1993, 1995) studied the nervous system, sensory organs, excretory system, and genital primordium of L_3 larvae. Endo and Trpis (1997) examined infective L_3 larvae of *O. volvulus* to elucidate the ultrastructure and the interrelations of the stoma, esophagus, intestine, and nervous system.

Microfilariae are readily available from human skin in basic epidemiologic studies; however, the infective third-stage larvae are acquired with greater difficulty from field-collected *Simulium* black flies in biting catches on human bait. Limited information is available on development of *O. volvulus* in the black fly hosts because maintaining the infected flies for 7–8 days in the laboratory is difficult. Documenting the development of *O.volvulus* in black flies is paramount to understanding the host–parasite relationship. Furthermore, this knowledge is essential for production of L_3 antigens for development of a vaccine against river blindness (Lange et al., 1993). Recently, larval stages that developed in black flies became available from procurement of infective larvae of *O. volvulus* that served as a source of antigen for vaccine development. In the present study, aspects of *O. volvulus* development in black flies, including their larval growth, morphology, and the duration of stages in *Simulium yahense* are described from Liberia. In addition, this study presents evidence for pathological consequences for the fly hosts.

MATERIAL AND METHODS

Rearing of black flies infected with *O. volvulus* microfilariae

Pupae of *Simulium yahense* were collected on branches and aquatic grass from Du River on the Firestone Rubber Plantation in Liberia; they were placed into food coolers and submerged in river water to maintain the original water chemistry and temperature during transportation to the Liberian Institute for Biomedical Research at Robertsfield. Pupae were then transferred into black plastic 50-L barrels that were sealed with lids containing a 10-cm hole that was covered with a fly cage. After 24 hr, flies started emerging from the pupae, and within 48 hr, fly emergence was complete. Emerging flies were separated by sex, and females were transferred into fly cages that were kept in the insectary at 26.0 ± 1 C. The constant insectary temperature, presence of running water on the bottom of the rearing cages, and a 25W bulb placed 20 cm above each cage resulted in cage temperatures of 25.5 C at the bottom and 27.5 C at the top. Between 24 and 72 hr after emergence, black flies were fed on volunteer donors infected with microfilariae of *O. volvulus*. Flies engorged with infected blood were transferred back to the cages in the insectary. Daily samples of infected flies were removed from rearing cages and dissected to recover the developing *O. volvulus* larvae. The dissected larval stages were observed, photographed, and measured with an Olympus BH2 microscope equipped with Nomarski interference contrast optics.

Cryopreservation of *O. volvulus* larvae in black flies

For further study and documentation of the development of *O. volvulus* larvae in *S. yahense*, daily samples of infected flies were cryopreserved by the method of Trpis et al. (1993). The cryopreserved material was shipped to Johns Hopkins University (Baltimore, Maryland), where the ampoules with flies were removed from liquid nitrogen and thawed by submerging in a water bath at 28 C. Flies were washed in Medium 199, transferred into a clean medium, and dissected under a microscope to recover the developing larvae from the fly's thoracic muscles. After dissection from *S. yahense*, the *O. volvulus* larvae were alive and appeared normal. The first- (L_1) and the secon-stage (L_2) larvae moved with typical, occasional jerky motion. The thawed infective (L_3) larvae displayed typical vigorous wriggling movements.

Preparation for SEM observation

Specimens were placed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8, for 2 hr at room temperature. Following chemical fixa-

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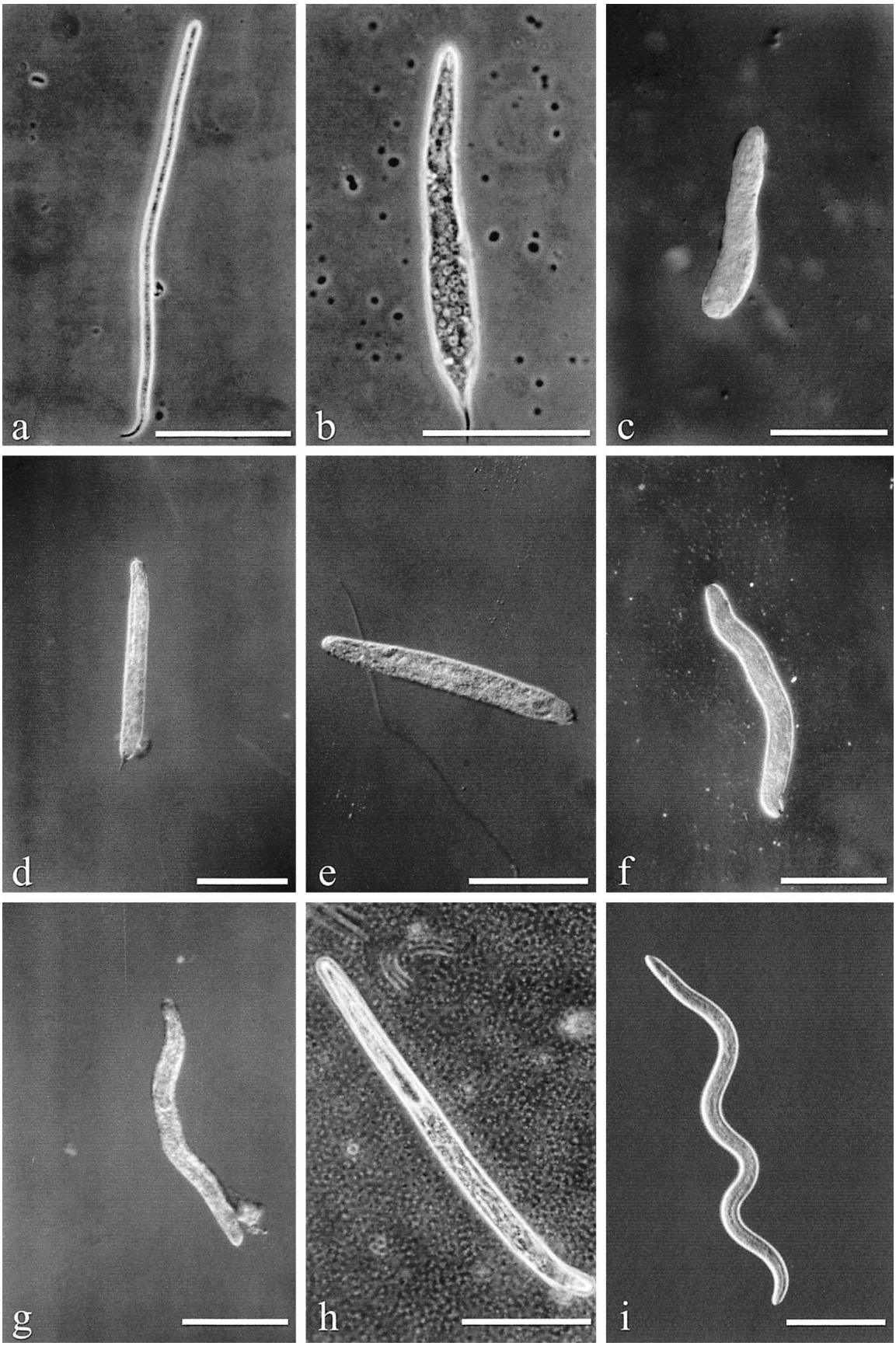
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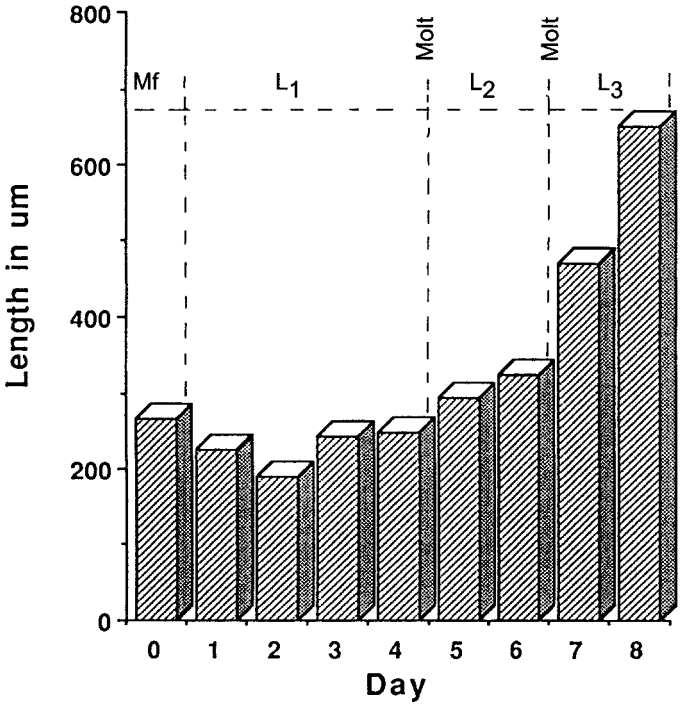

FIGURE 2. Graphic expression of larval growth by days and molting of *Onchocerca volvulus* in *Simulium yahense*.

tion, the specimens were rinsed 5 to 6 times in the phosphate buffer over a 1-hr period. Several of the specimens were also postfixed in 2% osmium tetroxide in phosphate buffer for 2 hr and subsequently rinsed twice in the buffer. After fixation, all specimens were dehydrated at 30-min intervals in a graded alcohol series, culminating with 3 changes in 100% ethanol. The dehydrated specimens were critical point–dried in a Tousimis Samdri 780A dryer using carbon dioxide as a transitional fluid. Dried specimens were mounted on stubs using Avery labels, coated with gold/palladium in a Hummer sputer coater, and viewed in either a Hitachi S-570 scanning electron microscope or a Hitachi S-4100 field emission scanning electron microscope.

RESULTS

Development and measurements of *O. volvulus* larval stages with light microscopy

Microfilariae (Mf) recovered from human skin snips measured 267 ± 19 μm. At this time, they migrated from the ingested blood in the fly gut into the thoracic muscles, where they transformed into the first larval stage (L_1) without molting. After the first 24 hr, most of the organisms were in the L_1 stage, which measured 226 ± 15 μm. On the second day, the L_1 larvae shortened to 171 ± 12 μm and became wider. At this time, their shape resembled a sausage, and they were designated the ''sausage stage'' (Fig. 1d–e). By day 3, lengths increased to 243 ± 8 μm and by day 4 to 269 ± 23 μm. Between day 4 and 5, larvae molted into the second stage (L_2), measuring 293 ± 32 μm on day 5 and 326 ± 45 μm on day 6. Toward the end of day 6, some larvae had reached the third stage, and on day 7, all were in the L_3 stage (Fig. 1i), which measured 471 ± 67 μm long. On day 8, the L_3 larvae measured 651 ± 38 μm. After molting to the third stage, the larvae left the thoracic muscles, either swimming freely in the hemolymph or migrating through various fly organs, including the head region, particularly the mouth parts. The daily development through three stages and time of moltings is depicted in Figure 2. The complete larval development from Mf to L_3 larva is depicted in (Fig. 1a–i).

SEM of *O. volvulus* larvae developing in the *S. yahense* vector

First-stage larva: In the first larval stage, the cephalic cap remains intact. The cap has a diameter of 2.6 μm and is equipped on 1 side with an elongated 0.6-μm hook. The mouth opening in the L_1 larva is in the center of the cephalic cap (Figs. 3, 4). Deep, regular cuticular ridges or annuli (0.1–0.2 μm) occur around the cephalic cap. Small globular protuberances occur scattered on the cephalic cup and in 3–5 concentric circles around the cephalic cap (Fig. 4). Toward the end of the fourth day, the L_1 larva begins the first molting, as indicated by loosening and shriveling of the cuticle (Fig. 4). The anal opening (Figs. 5, 6) is located laterally on the L_1 larva, approximately 9.0 μm from the base of the caudal appendix (Fig. 5). The caudal appendix, which is visible by light microscopy as a bristle, is depicted by SEM as a large terminal appendix (Fig. 7). Rows of cuticular folds (Fig. 8) are located bilaterally on the L_1 larva. The rest of the cuticular surface is smooth, without significant annulations.

Second-stage larva: Molting from the L_1 to the L_2 stage (Fig. 9) takes place by the end of day 4 and continues to day 6 (Fig. 2). The results of the first molt can be observed on Figure 10. The cephalic cap is lost, and a large stoma (st) is surrounded with elevated flops (Fig. 10). The cuticle loosens, indicating that the caudal appendix will be shed in the second molt; the caudal end of a young L_3 larva, visible through the unshed L_2 cuticle, appears to be blunt (Fig. 11). The cuticle sloughs (Fig. 12) and disintegrates, and the final phase of this process is characterized by a loosened cuticular network (Fig. 13).

Third-stage larva: By the end of day 6 and on day 7, the L_2 larva initiated the second molt. On day 7, L_3 infective larvae (Fig. 1i) can be transmitted by black flies during feeding on human hosts. The cephalic region of the L_3 larva has 8 papillae arranged in 2 circles (4 papillae in each circle) around the mouth opening (Fig. 14). In addition, 3 terminal papillae measuring 2.3, 1.1, and 1.9 μm are located on the tip of the posterior end of the L_3 larva (Figs. 15, 16). The nerve endings of the proximal and caudal papillae suggest that these structures have a tactile function (Endo and Trpis, 1997) which assists the L_3 larva in moving through the fly body and reaching the fly mouth parts, from where they can be transmitted into a human host. The amphids (Fig. 14a), which may sense the chemical environment (Endo and Trpis, 1997) of the fly inner body, also can direct the larvae toward

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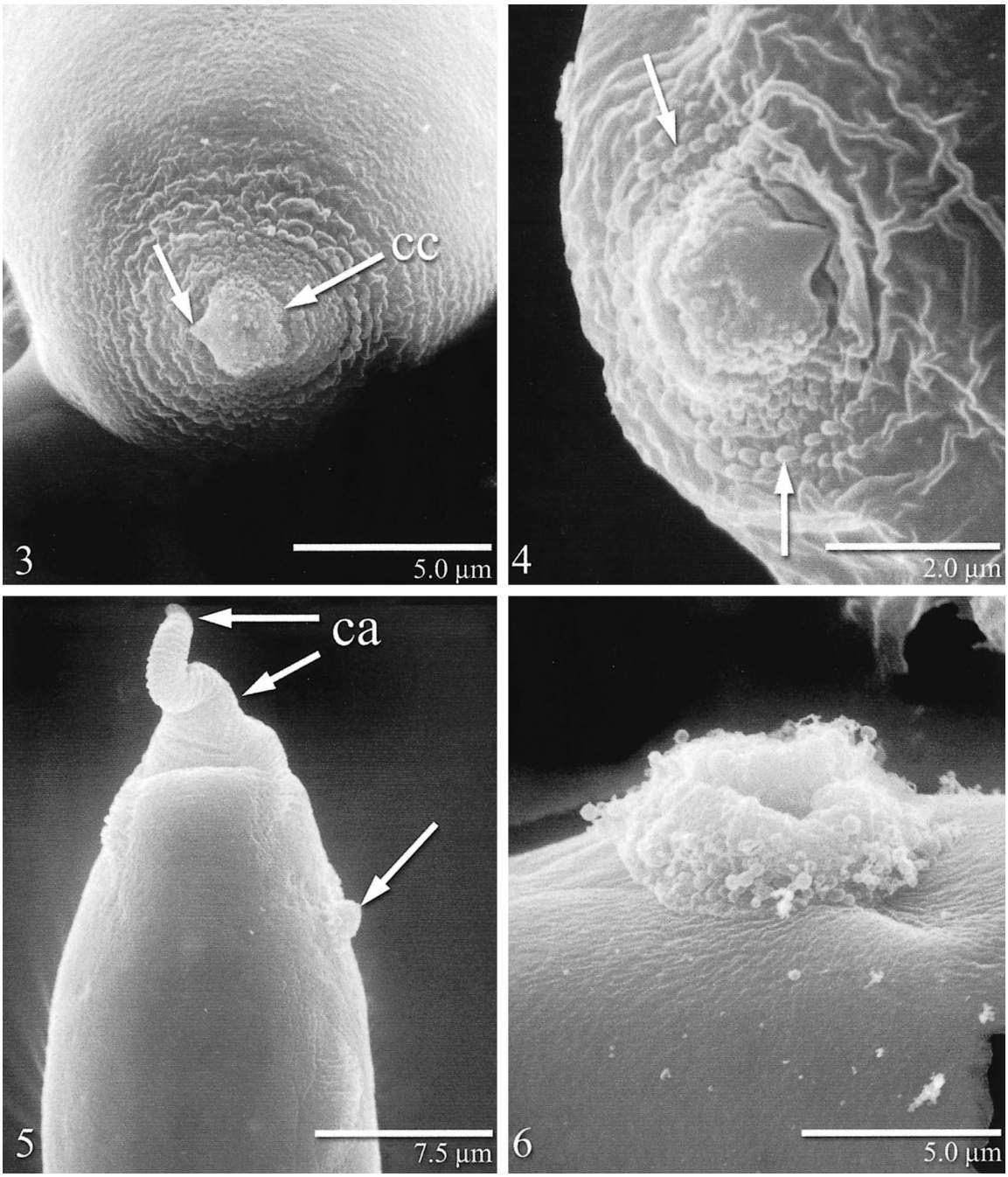
FIGURE 1. Photomicrographs of *O. volvulus* larvae developing in *S. yahense* black flies. (**a**) Microfilaria. (**b**) An early stage of L_1. (**c**) L_1 larva in the process of shortening. (**d, e**) L_1 larva in the ''sausage stage.'' (**f, g**) Larvae in the L_2 stage. (**h**) Early stage of L_3. (**i**) Typical serpentine movement of the infective (L_3) larva. Scale bars = 100 μm.



FIGURES 3–6. **3.** The proximal end of the first-stage larva (L_1); the cephalic cap (cc) with the sharp, pointed cephalic hook (arrow) shifted and pointed to one side. The cuticular ridges (annuls) are deep and irregular. **4.** Globular protuberances (arrows) are seen on the cephalic cap, at the base of the cephalic hook, and around the stoma. They are arranged around the cephalic cap in a semicircular fashion. **5.** The rectal plug (arrow) on L_2 is located on the left side near the base of the caudal appendix (ca). **6.** Detail of the rectal plug of the L_1.

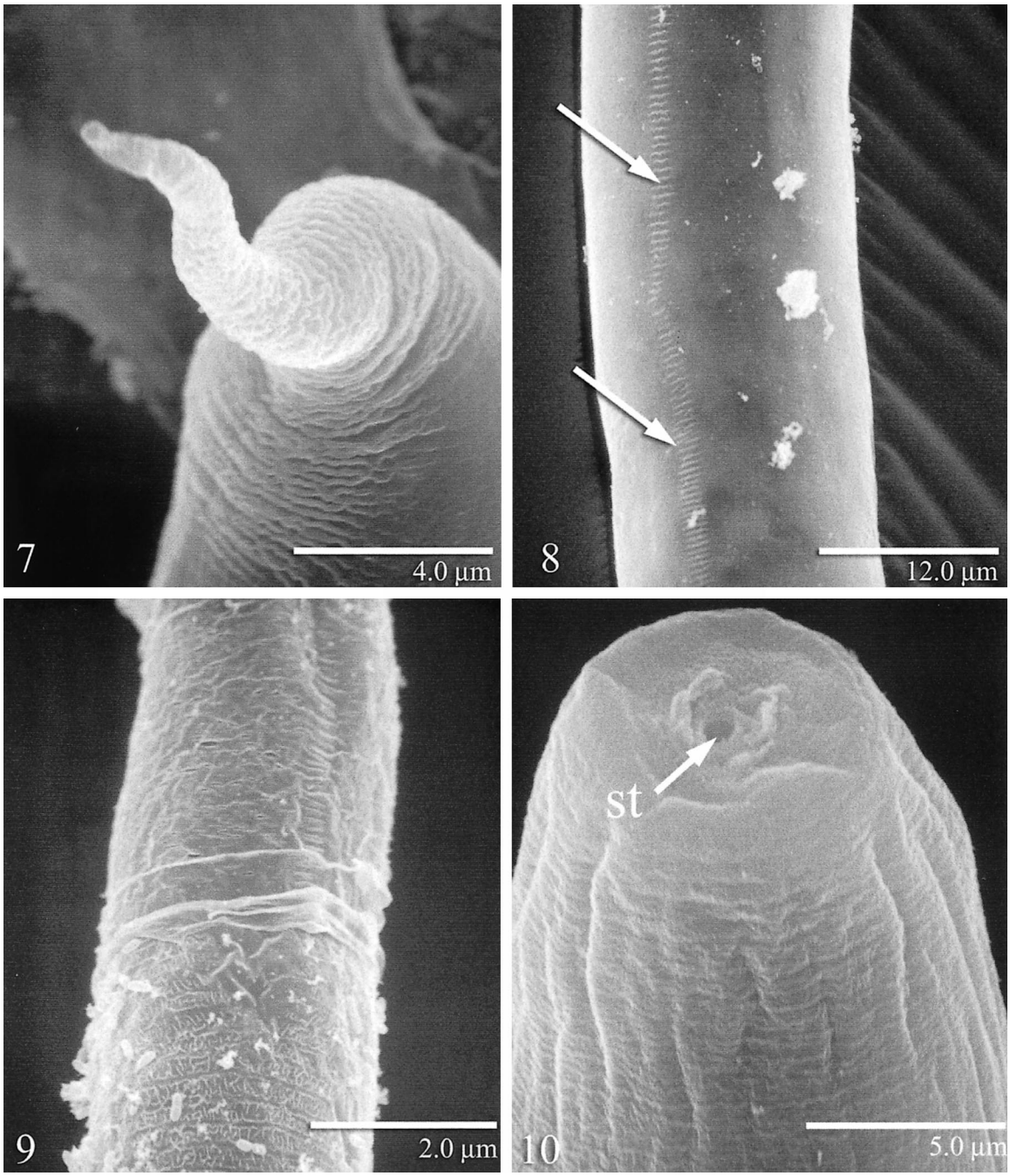
the fly mouth parts where saliva is released. After entering the human body, the L_3 larvae locate suitable sites in the dermis where onchocercal nodules can develop and where the L_3 larvae develop to the adult stage.

The anal opening is located at the distal end of the L_3 larva, 18.0 µm from the caudal tip (Fig. 15ao). A cuticular rosette measuring 5.0–5.8 µm surrounds the anus. The cuticle of the L_3 larva forms folds of horizontal circles apparent on Figure 15.

DISCUSSION

Changes in the development of the larval sensory organs

The amphidial sensory organs present in microfilariae (Franz and Schultz-Key, 1981) are not apparent in the L_2, but can be seen in the L_3, larvae. These sense organs may stimulate the L_3 larva to move toward the saliva released by a fly while feeding on a human host. Similarly, amphids present in Mf may assist

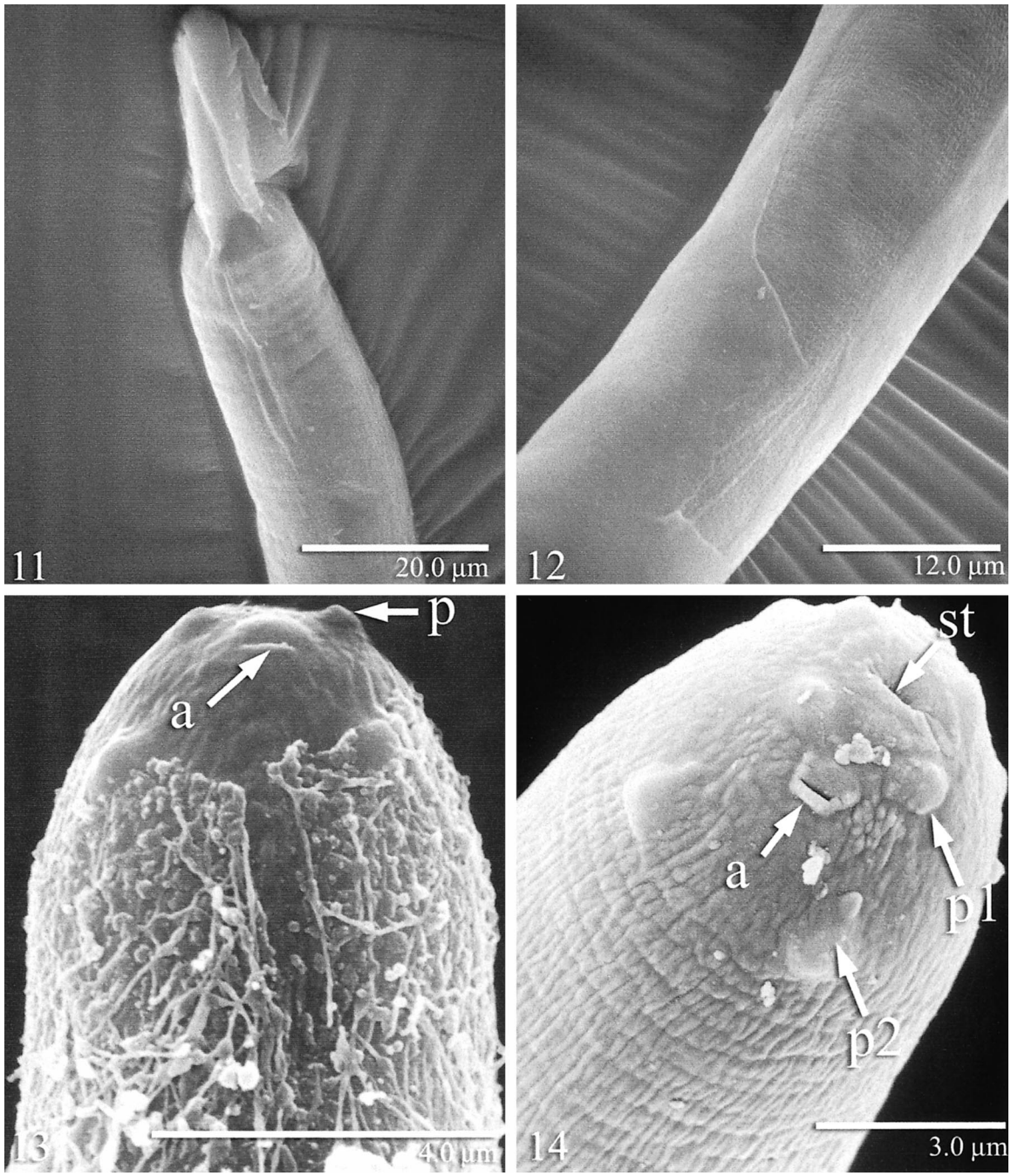


FIGURES 7–10. **7.** The caudal appendix of the L_1 from the ventral side. **8.** The lateral cuticular folding (arrows) of the middle part of the L_1 larva. The cuticular surface between lateral foldings has a smooth appearance. **9.** Molting from the L_1 to L_2 stage in progress. The old cuticle is loosened, and the lateral cuticular folding is evident. **10.** Proximal end of the L_2 larva. The stoma (st) is circular, surrounded by cuticular folds.

them in finding the way from the fly midgut into the hemocoel and to the thoracic muscles. After they reach the thoracic muscles, Mf become sedentary until development to the L_3 stage is completed. After the second molt, from the L_2 to L_3 stage, the amphids reappear, and a new set of sensory organs, the papillae, develop on the caudal end of the L_3 larva. The amphids and papillae may assist the larva in movement within the fly and help the L_3 to find a suitable place for development in human subdermal tissue into the adult stage.

Effect of variable and constant temperature on developing *O. volvulus* larvae in black flies

Uniform development of *O. volvulus* larvae in a population of *Simulium* sp. flies cannot be achieved when infected, by feeding on 1 host, and released even into the same habitat in the natural environment. Whereas most individuals of the developing larvae change into a successive higher stage, a few individuals can fall behind. This phenomenon occurs in nature

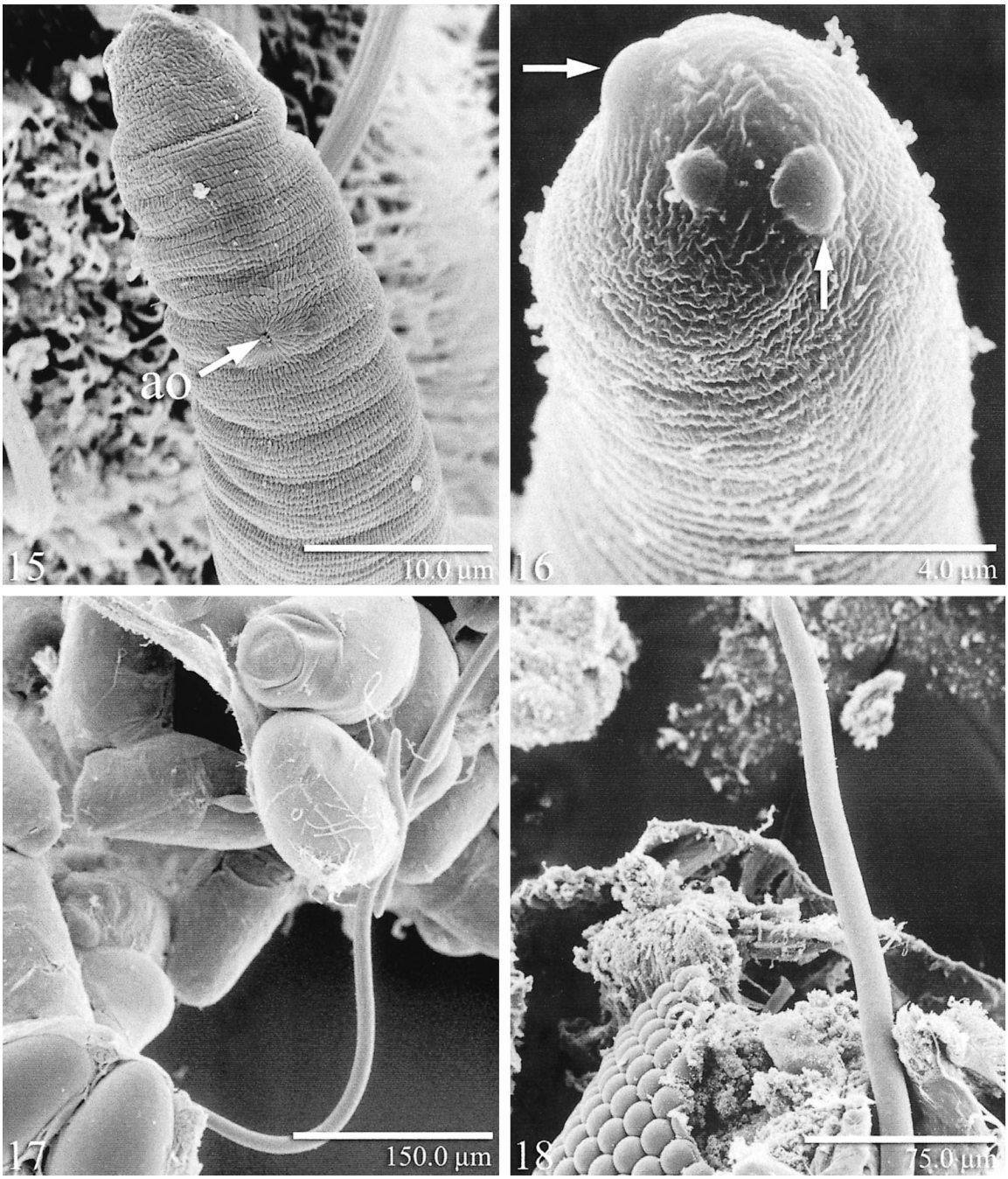


FIGURES 11–14. **11.** Molting from the L_2 to L_3 stage. The cuticle is loosened, and the caudal appendix is detaching from the new L_3 stage, which ends bluntly. **12.** Sloughing of the old cuticle during molt from the L_2 to L_3 stage. The new L_3 cuticle has the characteristic circular annulation. **13.** The loosened disintegrating L_2 cuticular network. The proximal end of the new L_3 larva has 2 amphids (a) (only 1 is exposed), and 8 sensory papillae (p) are arranged into 2 circles (only 4 are exposed). **14.** The cephalic end of the infective L_3 larva. The circular stoma (st), two circles of papillae (p1, p2), and 2 amphids (a) are located on opposite sides near the inner circle of papillae and between two papillae. The second amphid is not visible.

more often in flies infected in the dry season than in flies infected in the rainy season but can be observed even on larvae developing in flies kept at constant temperature. This may indicate that not all larvae in the thoracic muscles are supplied with nutrients uniformly or take them equally. It may also indicate differences in the genetic makeup of individual larvae.

The fate of epicuticle in molting

In molting from the L_1 to L_2 stage, the epicuticle was sloughed off, often in 1 piece, and reabsorbed within the fly or by the developing worms. In molting from the L_2 to L_3 stage, the cuticle gradually peeled off (Fig. 12) and, in some cases,

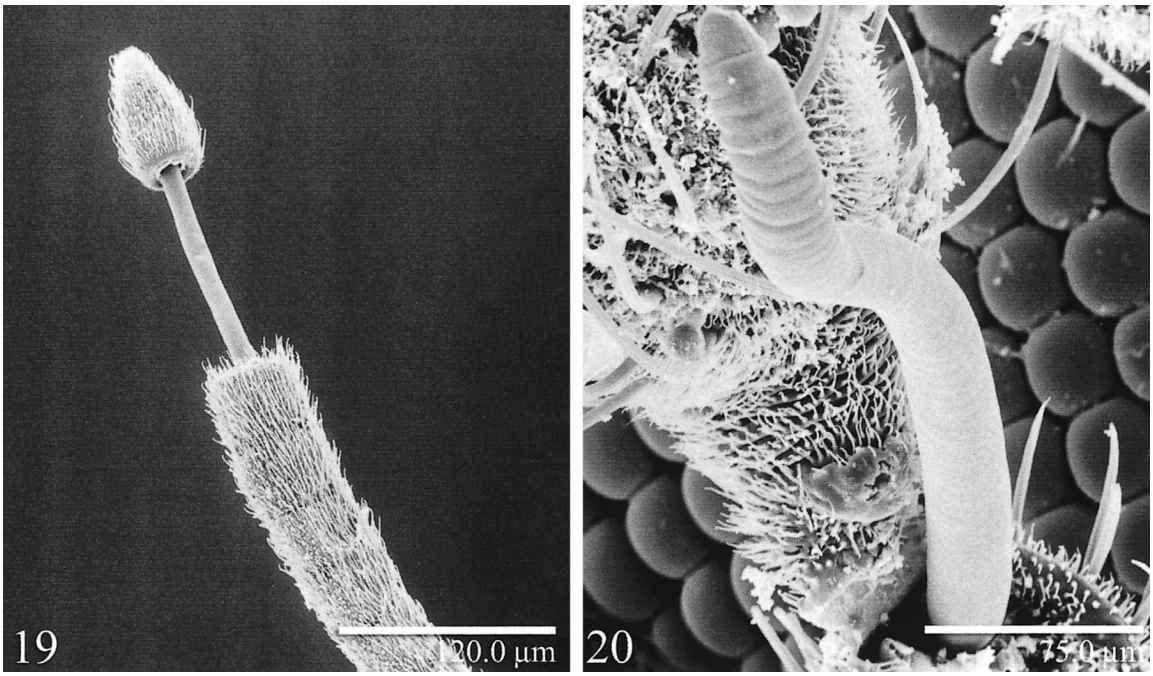


FIGURES 15–18. **15.** The caudal end of the L_3 larva with anal opening (ao). Three papillae are apparent on the tip of the caudal end. **16.** The distal end of the L_3 larva with 3 terminal papillae. **17.** Infective L_3 larvae found in the ovaries of *S. yahense* black flies. **18.** Infective L_3 larvae found in the head of *S. yahense*.

remained attached to the L_3 larva before gradually being reabsorbed before the L_3 larva left the thoracic muscle (Fig. 13).

Exiting the fly host and entering the definitive human host

After the L_3 larva migrates from the thoracic muscle into the celomic cavity by swimming in the hemolymph, they penetrate various fly organs. The random wandering of L_3 larvae through the fly body is an effort to escape their fly host. The fly mouth is the most natural opening for the L_3 larva to escape from the fly and be transmitted to the human host. Whether chemical signals are released prior to fly feeding is difficult to determine. However, if this were the case, the signal could assist the L_3 larvae in finding the site they could successfully escape from and enter the human



FIGURES 19–20. **19.** Infective larva in the fly antenna. The last antenna segment dislodged by the emerging L_3 larva. **20.** Infective L_3 larva escaping through a palp segment of *S. yahense*.

body. Such chemical signals could be sensed by the amphids in the L_3 stage when the fly starts to salivate, releasing saliva into the human body while imbibing blood.

Destruction of the fly organs by the L_3 larva

When the L_3 larvae leave the fly thoracic muscles, they swim in the hemolymph and often penetrate several fly organs, such as the ovaries (Fig. 17), the brain (Fig. 18), antennae (Fig. 19), and the palps (Fig. 20). The extent and severity of damage caused by L_3 larvae to the fly is not known but should be determined in the future because it affects vector survival and the intensity of the transmission of the infective larvae. The destructive power of the L_3 larva may result in serious consequences for the *Simulium* spp. flies, affecting their survival. When *O. volvulus* larvae reach the third stage in the fly, which takes place at day 7, fly mortality increases sharply, and often $<1\%$ of the flies infected with L_3 larvae survive to day 10.

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